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CLINICAL REVIEW

Significant biomarkers for the management of hepatocellular carcinoma

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Abstract Surveillance of hepatocellular carcinoma (HCC) is important for early detection. Imaging tests including computed tomography, magnetic resonance imaging and ultrasonography with or without various kinds of contrast medium are important options for detecting HCC. In addition to the imaging tests, various kinds of biomarkers including alpha-fetoprotein (AFP), lectin-bound AFP (AFP-L3) and protein induced by vitamin K absence or antagonist II (PIVKA-II) have been widely used to detect HCC and analyze treatment response. Recently, various kinds of novel biomarkers (proteins and miRNA) have been found to predict the malignancy potential of HCC and treatment response to specific therapies. Moreover, various combinations of well-established biomarkers and novel biomarkers have been tested to improve sensitivity and specificity. In practical terms, biomarkers that can be analyzed using peripheral blood samples might be more useful than immunohistochemical techniques. It has been reported that quantification of cytokines in peripheral blood and the analysis of peripheral immune subsets could be good biomarkers for managing HCC. Here, we describe the usefulness of and update well-established and novel biomarkers for the management of HCC.

Keywords AFP · PIVKA-II · AFP-L3 · miRNA · MDSCs · Tregs

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death worldwide and is caused by various kinds of chronic liver diseases. Risk factors for the incidence of HCC are chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), alcoholic and non-alcoholic fatty liver diseases, and other types of chronic inflammation of the liver [1, 2]. In Asia–Pacific countries except for Japan and Africa, the leading cause of HCC is CH-B. Worldwide, 53 % of HCC cases are related to HBV infection [3]. Although several mechanisms have been suggested to explain the formation of HCC in hepatitis B patients, the actual cause still remains unclear [4]. Integration of DNA into the host genome occurs during the early stages of clonal tumor expansion. HBx involvement has also been suggested to be involved in hepatitis B virus-related HCC. Recently, it was reported that HBx was related to a cancer stem cell [5]. In the USA, Europe, and Japan, the leading cause of HCC is CH-C [2, 6]. Recently, well-developed treatments have become available for CH-C and CH-B and >90 % of CH-C patients can achieve a sustained viral response. However, even eradicating hepatitis C virus in CH-C patients might not substantially reduce HCC in comparison to healthy subjects. Toyoda et al. [7] reported that the incidence of HCC was 1.2 % at 5 years and 4.3 % at 10 years, and patients with diabetes mellitus and those with an elevated FIB-4 index at SVR24 were at a higher risk of HCC after eradicating hepatitis C. Moreover, it has been reported that CH-B patients who were treated by nucleoside analogs are still at risk for HCC [8, 9]. Therefore, surveillance of HCC is important for early detection. Imaging tests including computed tomography (CT), magnetic resonance imaging (MRI) and ultrasonography (US) with or without various kinds of contrast medium are significant options for

detecting HCC [10]. In addition to imaging tests, various kinds of biomarkers including alpha-fetoprotein (AFP) [11], lectin-bound AFP (AFP-L3) and protein induced by vitamin K absence or antagonist II (PIVKA-II) have been widely used to detect HCC and analyze treatment response [12, 13]. Recently, various kinds of novel biomarkers (proteins and miRNA) have been found to predict the malignant potential of HCC and the treatment response to specific therapies [14–16]. Therefore, we should be familiar with well-established and novel biomarkers for managing HCC. Here, we summarize and update useful biomarkers for the management of HCC.

Alpha-fetoprotein

AFP is a protein discovered by Bergstrand and Czar [17]. AFP is a 70 kDa glycoprotein consisting of 591 amino acids. It acts as a transporter molecule for several ligands [18] such as fatty acids, phytoestrogen heavy metals, and retinoids; however, its exact biological function is not clear. AFP is synthesized by the yolk sac during early fetal life and later in life by the fetal liver and certain tumors. AFP increases in pregnant women, but hardly appears in normal adults. Therefore, AFP is a tumor marker for various types of cancer, e.g., hepatocellular carcinoma, gastric carcinoma, lung cancer, and testicular carcinoma [19–21]. Tatarinov first reported high levels of serum AFP in HCC patients [22]. Today, AFP has come to be used as a common tumor marker for HCC (Table 1). Trevisani et al. [23] reported AFP levels in patients with 170 HCCs and 170 chronic liver diseases (CLDs) in a case−control study. In this study, they suggested an AFP cut-off of 16 ng/ml, which resulted in a sensitivity and specificity of 62.4 and 89.4 %, respectively; a cut-off of 200 ng/ml resulted in a sensitivity and specificity of 22.4 and 99.4 %, respectively. They suggested the best cut-off levels for AFP would be in the range of 16−20 ng/ml. Other studies [24–26] reported similar sensitivity and specificity values at a cut-off of 20 ng/ml; however, false positives appear at a cut-off level of 20 ng/ml because AFP rises in patients with cirrhosis. AFP has high specificity but insufficient sensitivity; this means that 40 % of HCCs are overlooked by serum AFP level alone. For HCC screening, AFP needs to be combined with imaging or other tumor markers. Ishii et al. [26] reported the sensitivity and specificity of a combination of AFP (cut-off value of 40 ng/ml) and PIVKA-II (cut-off value of 80 mAU/ml) to be 65.5 and 85.5 %, respectively. Combined tests of AFP and PIVKA-II showed a higher sensitivity than AFP alone; however, the sensitivity was still insufficient. The most widely used method for HCC surveillance are AFP and abdominal US. US has many advantages including being non-invasive and enabling real-time observation; however, there have been a variety of results in the application of US for HCC surveillance. Sheraman et al. [27] reported sensitivity and specificity of 71.4 and 93.8 %, respectively. On the other hand, Gambarin-Gelwan et al. reported sensitivity and specificity of 58 and 94 %, respectively. Differences in sensitivity were considered to be due to operator skill and patient size. However, in patients with cirrhosis, the cost−benefit of performing liver US and AFP every 6 months is very high [28]. The combination of AFP and US provides an increase of 6–8 % in detection of HCC; however, false positives also increase, and cost-effectiveness is poor. Accordingly, the AASLD and EASL guidelines do not recommend AFP for surveillance because of the high false positive rate [29, 30]. As sensitivity may differ depending on etiology [31], it may not be appropriate for surveillance use.

AFP is used for HCC staging, e.g., the Cancer of the Liver Italian Program (CLIP) score. The CLIP score is made up of four independent predictive factors—Child–Pugh stage, tumor morphology, AFP and portal vein thrombosis. AFP is used as a categorical variable with a cut-off of 400 ng/ml. The CLIP score consists of liver function and tumor characteristics and is very useful for predicting convalescence.

Table 1 Sensitivity and specificity of AFP for HCC screening

Author	Year	Country	No. of patients	Cut-off (ng/ml)	Sensitivity (%)	Specificity (%)
Trevisani et al. [23]	2001	Italy	340 CLD (170 HCC)	16	62.4	89.4
				20	60.0	90.6
				100	31.2	98.8
				200	22.4	99.4
				400	17.1	99.4
Cedrone et al. [24]	2000	Italy	350 CLD (72 HCC)	20	55	88
Gambarin-Gelwan et al. [25]	2000	USA	106 CLD (19 HCC)	20	58.0	91.0
				50	47.0	96.0
Ishii et al. [26]	2000	Japan	734 CLD (29 HCC)	20	61.2	78.3

Lectin-bound AFP (AFP-L3)

AFP is further subdivided into three different forms of glycoforms such as AFP-L1, L2 and L3, according to their binding ability to the lectin *Lens culinaris* agglutinin (LCA). AFP-L1, as a non-LCA-bound heterogeneity, is the major form in benign liver diseases. AFP-L3, as an LCA-bound heterogeneity, has α1–6 fucose residue appended to *N*-acetylglucosamine of the reducing end. It is associated with a large mass of cancer tissue, poor differentiation, and malignant features [12, 32]. Oka et al. [33] examined the cut-off value of AFP-L3 in 388 patients with newly diagnosed HCC. They reported that a cut-off value of >15 % AFP-L3 reflected HCC characteristics. Some clinical researchers have indicated that AFP-L3 has a sensitivity ranging from 75−96.6 % and a specificity ranging from 90−92 % when using a cut-off of 15 % [12, 34, 35]. These data showed that AFP-L3 has a higher sensitivity than AFP; however, these studies did not necessarily have large numbers of patients. In fact, it is possible that the sensitivity and specificity of AFP-L3 are influenced by the concentration of AFP. Nouso et al. [36] reported that the sensitivity of AFP-L3 in HCC patients with low AFP (<20 ng/ml) was 51.5, 13.3, and 8.7 % with cut-off values of 5, 10, and 15 %, respectively. On the other hand, Leerapun et al. [37] reported that the sensitivity of AFP-L3 in HCC patients with total AFP of 10–200 ng/ml was 71 % at a cut-off value of 10 %. However, with regard to the combination of AFP-L3 and AFP, AFP alone showed no superiority for the diagnosis of HCC in a meta-analysis by Hu et al. [38].

Protein induced by vitamin K absence or antagonist II

PIVKA-II was discovered in 1984 by Liebman et al. [39]. PIVKA-II, also known as des-gamma-carboxyprothrombin (DCP), represents an abnormal product of liver carboxylation during the formation of thrombogen that acts as an autologous mitogen for HCC cell lines [13, 40]. Cui et al. [41] reported sensitivity and specificity of PIVKA-II (cut-off value of 40 mAU/ml) in discriminating between HCC and cirrhosis to be 51.7 and 86.7 %, respectively, while the combined test of PIVKA-II and AFP showed a sensitivity of 78.3 %, which is higher than that of PIVKA-II (51.7 %) and AFP alone (56.7 %). Sensitivity and specificity in other studies are similar (Table 2) [42–44]. In another study, PIVKA-II showed a sensitivity of 53.3 % and specificity of 85.6 %, while the combined tests of PIVKA-II and AFP showed a sensitivity of 78.3 %, which is higher than that of PIVKA-II (53.3 %) and AFP alone (58.3 %) [45]. However, in a meta-analysis by Hu et al. [38], PIVKA-II was not statistically better than AFP, although the combined measurement of PIVKA-II and AFP was superior to AFP alone. Generally, the diagnostic sensitivity of PIVKA-II was weaker compared with AFP when HCC tumor size was <3 cm, while it was stronger than AFP when HCC tumor size was >5 cm [46]. Hashimoto et al. [31] reported that the AFP increased in non-alcoholic steatohepatitis-related HCC (NASH-HCC), and PIVKA-II increased in hepatitis C virus-related HCC. They suggested that the sensitivity of AFP and PIVKA-II might change depending on etiology. Masahiro et al. suggested that the treatment procedure was a significant factor for survival in patients with PIVKA-II >100 mAU [47]. PIVKA-II is more likely to be elevated in patients with more advanced HCC, e.g., larger tumor, vascular invasion or metastasis. Therefore, PIVKA-II is being focused on as a candidate for new recipient selection criteria for living donor liver transplantation (LDLT). At present, the Milan selection criteria (single nodule ≤5 cm or ≤3 nodules, all ≤3 cm) are generally used for LDLT [48]. PIVKA-II has been applied for LDLT cases that deviate from the Milan criteria. However, the presence of vascular invasion suggested a poor prognosis after liver transplantation for HCC. Beyond Milan criteria, it is important to exclude cases with vascular invasion. Accordingly, some research groups use the PIVKA-II value [49, 50]. A research group in Kyushu University set new criteria as both a PIVKA-II level of <300 mAU/ml and a tumor size of <5 cm [49]. The research group in Kyoto University added the number of tumor nodules into their criteria and a PIVKA-II level of ≤400 mAU/ml and ≤10 nodules, all with a size of not >5 cm [50]. In addition, these studies suggested a correlation between a higher level of PIVKA-II and the presence of microvascular invasion in resected HCC. A further validation study is necessary to confirm the usefulness of including PIVKA-II in the new criteria.

Table 2 Sensitivity and specificity of PIVKA-II for HCC screening

Author	Year	Country	No. of patients	Cut-off (mAU/ml)	Sensitivity (%)	Specificity (%)
Cui et al. [41]	2002	China	60	40	51.7	86.7
Yamamoto et al. [42]	2009	Japan	714	40	55.9	99.8
Marrero et al. [43]	2009	USA	419	150	74	70
Sterling et al. [44]	2009	USA	74	40	76	58

Table 3 Novel cytokine and protein biomarkers

	Author	Year	Country	Sample size	Clinical relevance
IGF2BP1	Gutschner et al. [54]	2014	Germany	60	Carcinogenesis
MyD88	Liang et al. [15]	2013	China	110	Poor survival, metastasis
c-Met	Lee et al. [55]	2013	Korea	287	New potential drug target
VEGF	Tsuchiya et al. [14]	2014	Japan	63	The prediction of sorafenib treatment response
MDSC	Arihara et al. [57]	2013	Japan	123	Poor survival
MCP-1	Wang et al. [64]	2013	Singapore	126	HCC diagnosis

Novel cytokine and protein biomarkers

Increasing numbers of novel cytokines and protein biomarkers have been reported following improvements in detection technology [51, 52]. High-sensitivity enzyme-linked immuno sorbent assay (ELISA) and high-throughput detection methods are being used to find biomarkers [53]. Moreover, understanding the molecular pathogenesis of HCC could contribute to revealing cytokines and proteins that may represent useful biomarkers for HCC. In addition, several genetic and epigenetic alterations, e.g., loss of tumor suppressor gene expression [tumor protein p53 (TP53), phosphatase and tensin homolog (PTEN), retinoblastoma protein (RB)] as well as activation of oncogenes [c-myc protein (c-MYC), MET proto-oncogene (MET), B-Raf proto-oncogene (BRAF), Ras protein (RAS)] are involved. One group analyzed the role of RNA-binding proteins which regulate tumor suppressor and oncogene expression at the post-transcriptional level. They found that RNA-binding protein insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) is an important protumorigenic factor in liver carcinogenesis [54]. Another group reported that the expression of myeloid differentiation primary response gene 88 (MyD88) could enhance activation of nuclear factor (NF)-kappaB and p38/extracellular signal-regulated kinase without Toll-like receptor/interleukin-1 receptor signaling. The expression of MyD88 was significantly higher in HCC tumors than in adjacent nontumor tissues. In particular, high expression of MyD88 was found in HCCs with late tumor stage ($P = 0.029$). Patients with high MyD88 staining revealed a higher recurrence rate (65 vs 40 %; $P = 0.008$). Kaplan–Meier analysis showed that recurrence-free survival (RFS; $P = 0.011$) and overall survival (OS; $P = 0.022$) were significantly worse in patients with high MyD88 staining [15]. On the other hand, the expression level of a molecular target protein related to the molecular pathogenesis of HCC could not be used as a biomarker for the prognosis of HCC patients [55]. It has been reported that c-*N*-Methyl-*N*′-nitro-*N*-nitroso-guanidine HOS transforming gene (c-Met) is a new potential drug target for the treatment of HCC patients; 27.9 % of HCC patients had c-Met over-expression. However, c-Met over-expression was not associated with Edmondson grade, tumor size, microvascular invasion, major portal vein invasion or tumor stage. Moreover, c-Met expression levels did not affect RFC or HCC-specific survival [55]. In comparison to immunohistochemical techniques, quantification of serum proteins could be more useful for managing HCC patients, since repeated examination and sequential analysis could be carried out during HCC treatment. Tuchiya et al. [14] reported that a decrease in the plasma vascular endothelial growth factor (VEGF) concentration at 8 weeks after starting sorafenib treatment might predict a favorable overall survival in advanced HCC patients. VEGF receptor is a sorafenib target. Therefore, the results of this study shoud be predictable. In addition to serum cytokines, the frequency of specific immune subsets including myeloid-derived suppressor cells (MDSCs) and regulatory T cells could be a useful biomarker for managing HCC [56, 57]. In addition to HCC patients, chronic hepatitis B, chronic hepatitis C and NASH patients have distinct immune cell profiles [58–63]. Therefore, for HCC, we should also consider background liver diseases. Moreover, novel biomarkers together with well-established biomarkers could improve diagnostic accuracy. It has been reported that the combination of AFP + MCP-1 (area under the curve [AUC] 0.974) showed significantly superior discriminative ability compared to AFP alone (AUC 0.942; $P < 0.001$) [64]. We should consider various combinations to improve the accuracy of biomarkers (Table 3).

Novel microRNA (miRNA) biomarkers

miRNAs are evolutionarily conserved small noncoding RNAs involved in the regulation of gene expression and protein translation. Many studies have indicated that miRNA deregulation could induce the onset and progression of cancer. Moreover, many groups reported that specific miRNA expression in various human cancers including hepatocarcinogenesis is closely associated with diagnosis and prognosis. Several groups reported that miR-122 might have an important role in the aggressive characteristics of

Table 4 Novel microRNA (miRNA) biomarkers

	Author	Year	Country	Sample size	Expression of miRNA	Clinical relevance
miR-122	Fan et al. [65]	2011	China		Down-regulated	Diagnostic marker, new potential drug target
	Kojima et al. [66]	2011	Japan			
miR-101	Fu et al. [67]	2013	China	25	Down-regulated (HCC tissues) Up-regulated (serum)	Biochemical marker of HBV-related HCC
miR-139	Li et al. [68]	2014	China	31	Down-regulated	Diagnostic marker, prognostic factor
miR-18b	Murakami et al. [69]	2013	Japan	110	Up-regulated	Poor survival
miR-221	Li et al. [70]	2011	China	46	Up-regulated	Poor survival

HCC [65, 66]. One group reported that the miR-122/CUX1/miR-214/ZBTB20 pathway could regulate AFP expression [66]. Another group reported that miR-122 plays an important role in HBV-related hepatocarcinogenesis by targeting N-myc downstream-regulated gene 3 [65]. The significance of circulating miR-101 has also been reported in HBV-related HCC. The expression of serum miR-101 in patients with HBV-related HCC was significantly higher than in healthy controls, and this increase correlated with hepatitis B surface antigen positivity, HBV DNA level, and tumor size [67]. Another study reported that miR-139 was downregulated in HCC patients. Receiver operating characteristic analysis of plasma miR-139 yielded an AUC of 0.764 with a sensitivity of 80.6 % and specificity of 58.1 % in differentiating between HCC and CH-B. Moreover, the combination of miR-139 and AFP improved the differentiating power [68]. Murakami et al. reported that miR-18b expression is an important marker of cell proliferation and cell adhesion, and is predictive of clinical outcome. After surgical resection, HCC patients with high miR-18b expression had a significantly shorter relapse-free period than those with low expression [69]. Moreover, it has been reported that a high level of miR-221 expression was correlated with tumor size ($P < 0.001$), cirrhosis ($P = 0.003$) and tumor stage ($P = 0.016$). In addition, the OS rate of the high miR-221 expression group was significantly lower than that of the low miR-221 expression group [70]. Increasing numbers of miRNAs could be significant biomarkers for the management of HCC (Table 4) [71–74].

Concluding remarks

The clinical significance of classical biomarkers (AFP, AFP-L3 and PIVKA-II) has been proved by many studies. However, the sensitivity of these biomarkers to detect small HCCs is not satisfactory. Novel biomarkers might contribute to better management of HCC. Moreover, various combinations of classical biomarkers and novel biomarkers should be tested to improve the sensitivity and specificity for detecting small HCCs and analyzing the prognosis of HCC.

Disclosures

Conflict of Interest: Yasuteru Kondo, Osamu Kimura and Tooru Shimosegawa declare that they have no conflict of interest.

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